We present a novel multi-confocal Fluorescence Correlation Spectroscopy (mFCS) technique that allows simultaneous FCS measurements in different locations within a cell. Standard FCS experiments are usually limited to one observation volume, so that information can only be obtained from one position at a time. In contrast, mFCS makes it possible not only to monitor fast temporal and spatial changes in the dynamics of cellular proteins, but also to increase the amount of data collected per measurement and thus reducing the time necessary to produce statistically significant results. Our mFCS technique takes advantage of a Spatial Light Modulator (SLM) to create several distinct observation volumes at a time. Parallel detection is performed using an Electron-multiplied CCD camera, where pixels act as pinholes for confocal detection. We were able to show that the spatial resolution and the sensibility of our mFCS system is close to that of an classical FCS setup. Employing a special camera readout mode, a temporal resolution of 14 µs is reached, which is adapted to the dynamics of most cellular proteins. The mFCS technique is applied to study the cellular response to thermal stress, by monitoring Heat Shock transcription Factor 1 (HSF1), which is a key regulator of heat shock response. Conducting experiments on living cells, we observed clear changes in the dynamics of HSF1 when heat shocking: its diffusion slows down, together with an increase in the bound fraction and in the residence time.

1003-Pos Board B789

Real-Time Tracking of Lanthanide Ion Doped Upconverting Nanoparticles in Living Cells

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Lanthanide ion-doped upconverting nanoparticles (UCNPs), which emit in the visible range upon absorption of NIR photons, have attracted great attention in the area of biological imaging owing to their unique properties. First, two-photon upconversion of NIR excitation to the emission of a visible photon is so efficient that a tiny CW laser with the output of tens of milliwatts is sufficient as the excitation source. Second, by employing NIR excitation, one can suppress cellular autofluorescence, hardly induce photodamage to cells, and achieve relatively deep penetration into tissues. Finally, UCNPs exhibit neither photoblinking nor photobleaching, and their cytotoxicity is very low. As a result, UCNPs became one of the most promising nanoparticle systems for biological imaging and there are continuing efforts to improve their properties (e.g., increasing luminescent intensity and reducing the particle size) by designing new synthetic strategies. In this study, we demonstrated the benefits of using UCNPs as the probe for real-time imaging and particle tracking in living HeLa cells. Combined with the low cytotoxicity and photostability of UCNPs, NIR excitation enabled uninterrupted long-term imaging of living cells. For the first time, we obtained real-time images of endocytosed UCNPs at the single vesicle level for 6 h continuously at the rate of 20 frames sec^{-1}. The dynamics of particle transport was composed of multiple phases within a single trajectory including the active transport by motor proteins such as dyneins and kinesins.

1004-Pos Board B790

Photoswitchable Biocompatible Polymer Dots Doped with Diarylethene

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Molecular photoswitches can be employed for the study of protein trafficking in living cells and applications in optical memories. Especially, to switch fluorescence, fluorescence quenching mechanism via energy or electron transfer is one of the most fundamental pathways to realize the system of photoswitching. In order to achieve fluorescence photoswitching, photochromic compounds such as diarylethene have been used to toggle fluorescence on and off. For example, photochromic diaryethene induces absorption changes upon light irradiations via cyclization reaction, which would trigger the fluorescence toggling. On the other hand, polymer dots (P-dots) is one of the promising fluorescent probes for the biological applications. We assumed that doping diarylethene into P-dots would realize fabrication of photoswhitchable P-dots via energy transfer mechanism between fluorescent polymer and diarylethene. In this study, we synthesized photoswitchable P-dots doped with diarylethene to toggle the fluorescence back and forth via energy transfer mechanism. We also tried to apply synthesized photoswitchable P-dots toward biological imaging. First, we examined the photoswitching properties with absorption and fluorescence measurements. Fluorescence of P-dots was dramatically quenched upon photoirradiation with UV light and recovered after visible light irradiation. Those photoswitching processes were reversible and could go through at least 5 cycles. We are now applying photoswitchable P-dots synthesized as mentioned above to biological imaging. Details will be discussed at the meeting.

1005-Pos Board B791

Back-Scattered Detection Provides Viable Signals in Many Conditions

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Precision position sensing is required for many microscopy techniques. One promising method, back-scattered detection (BSD), provides position sensing at the level of several picometers, and is compatible with platforms that have restricted optical access (e.g. magnetic tweezers, atomic force microscopy, and microfluidics). However, widespread adoption of BSD may be limited by recent theoretical modeling that predicts diminished signals under certain conditions. In BSD the position of a micron-sized bead is measured by back-scattering a focused laser off the bead and imaging the resulting interference pattern onto a detector. Theoretical modeling of the detector response assumes the bead acts as a Mie-Debye scatterer and creates a first order interference pattern in the back-focal-plane of the collection lens. According to this Mie-Debye scattering model the BSD signal reverses sign many times for bead radii between 100 nm and 2000 nm and that for some radii (e.g. 1000 nm) the BSD response would be vanishingly small, limiting the applicability of BSD. We directly measured the BSD response while varying the experimental conditions, including bead radius, medium refractive index, and numerical aperture of the objective. Contrary to the proposed theory, we find that the signal increases with bead radius. Furthermore, the signal sign does not fluctuate, as predicted, over the tested parameters of radius, numerical aperture, and medium refractive index. We conclude that BSD provides a viable signal in a plurality of conditions.

1006-Pos Board B792

Use of Fluorescent Sphingolipid Precursors for Biophysical Studies of Sphingolipids

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Sphingolipids are one of the major components of cell membranes and also play critical roles in cell signaling. Many studies of disrupted cells have expanded our understanding of sphingolipid metabolism and function. Nonetheless, investigations of dynamic sphingolipid events, such as trafficking, diffusion, and organization in cell membranes, require observation of fluorescent sphingolipid analogs within living cells. However, sphingomyelin or ceramide analogs that contain a fluorophore-labeled N-acyl fatty acid cannot be used to track sphingosine or sphingosine-1-phosphate in cells. Additionally, the catabolism of these fluorescent sphingolipids may also result in fluorophore incorporation into glycerolipid species. Though various fluorescent sphingosine analogs have been developed that permit the study of sphingosine and its metabolites, poor photostability of the fluorophores limits long term data collection. Here, we report the use of fluorescent sphingolipid precursors in which a borondipyrromethene (BODIPY) fluorophore is incorporated into the sphingosine backbone. The enhanced photostability of the BODIPY fluorophore improves the ability to observe dynamic sphingolipid events. The fluorescent sphingosine analogs are incorporated into cells by addition to the cell culture media. To verify metabolic incorporation of the fluorescent sphingosine into cellular sphingolipids, lipid extracts from labeled cells were analyzed by thin layer chromatography and mass spectrometry. We demonstrate that the fluorescent analogs of sphingosine can be used to study dynamic events of sphingolipids, such as transport and trafficking.

1007-Pos Board B793

Exploiting Fluorescence Lifetime Plasticity in Flim: Target Molecule Localisation in Cells and Tissues

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The mechanisms of drug-receptor interactions and the controlled delivery of drugs via biodegradable and biocompatible nanoparticulate carriers are active research fields in nanomedicine. Many clinically used drugs target G-protein coupled receptors (GPCRs) due to the fact that signaling via GPCRs is crucial

in physiological and pathological processes and thus central for the function of biological systems. We describe a fast and reliable ratiometric Fluorescence Lifetime Imaging Microscopy (rmFLIM) approach to analyze the distribution of protein-ligand complexes in the cellular context.[1] Binding of the fluorescently labeled antagonist naloxone to the G-protein coupled μ-opioid receptor is used as an example. To show the broad applicability of the rmFLIM method we extended this approach to investigate the distribution of polymer-based nanocarriers in histological liver sections.

References

(1) Boreham et al. (2011) DOI: 10.1021/ml200092m.

1008-Pos Board B794

Second Harmonic Generation Correlation Spectroscopy for Nanocrystal Characterization

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Many early trials at protein crystallization produce large amounts of sub-diffraction limited crystals. These nanocrystalline showers are challenging to quantitatively characterize by conventional optical methods; however, they can offer important indicators for improving crystallization conditions. Additionally, the advent and availability of ultrafast X-ray free-electron lasers now allows single-pulse diffraction from individual protein nanocrystals for structure determination. However, these and other applications of nanocrystals currently suffer major bottlenecks in sample characterization, limiting their broader utility. Second harmonic generation correlation spectroscopy (SHG-CS) is being developed to address this key characterization need. Under tight focus and high laser intensity, highly-ordered (crystalline) material lacking inversion symmetry; including the vast majority of protein crystals but not simple salt crystals, amorphous protein, solvents, etc.; allow for second harmonic generation, the frequency doubling of light. This provides a way to selectively track crystalline protein particles in solution. The size of the particles can be determined by taking advantage of the fact that the particles diffuse through solution. The amount of time spent in a particular pixel along with the size of the pixel allows diffusion constants to be extracted by autocorrelation. Although in principle, SHG-CS is similar to fluorescence correlation spectroscopy, practical challenges arising from the use of high average power (>100 mW) require constant motion of the beam. The coherence of SHG can also present unique challenges and potential opportunities compared to fluorescence-based correlation methods.

1009-Pos Board B795

New Voltage Sensitive Dyes

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To improve the photostability of voltage sensitive dyes (VSDs) we have explored structural modifications such as fluorination, trifluoromethylation, cyanation, and rigidification, on the classic ANEP (aminonaphthylethenylpyridinium) chromophore. Some new dyes show significantly improved photostability in confocal imaging of brain slices and in a photobleaching assay with a suspension of lipid vesicles. Most show high sensitivities to membrane potential changes when tested on a voltage-clamped hemispherical lipid bilayer (HLB) apparatus. The modifications also induce large spectral shifts, up to 50 nm in either blue or red direction, depending on the nature and position of modification. The high sensitivity and photostability of the new VSDs have already produced new applications, such as imaging of electrical signals in single dendritic spines. Because of the range of excitation and emission wavelengths covered by this array of new VSDs, they may be especially useful in multiplexed imaging of membrane potentials together with other fluorescent indicators in live cells and tissues. (Supported by NIH grant EB001963).

1010-Pos Board B796

Compressive Mechanics of Hyaluronan Brushes - A Study with a Combined Colloidal Probe AFM/RICM Setup

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Around many biological cells, in the transition zone between the plasma membrane and the extracellular matrix, lies the micrometer thick pericellular coat (PCC): hyaluronan (HA), a long and flexible, charged glycosaminoglycan and HA-binding proteins self-organize into a hydrated gel-like coat which serves crucial mechanical functions of the cell. For a thorough investigation of the physical principles underlying the biological functions of these coats and provided the instrumental limitations in studying these highly hydrated systems in vivo, model systems are useful.

Atomic force microscopy (AFM) is a widely used analytical approach to determine the behavior of molecules or thin films under mechanical force. Colloidal probe reflection interference contrast microscopy (RICM) is an established microinterferometric technique to determine the thickness of soft hydrated films. In this study we combine colloidal probe AFM and RICM to investigate the mechanical properties of a well-defined model system of the pericellular coat: films of hyaluronan that is grafted to supported lipid bilayers (Richter et.al. 2007, JACS, 129:5306-7). The combination provides interaction forces as a function of the absolute distance between the two approaching surfaces, information that cannot easily be obtained with either technique alone. We quantify the thickness of HA films, and their resistance to compression forces as a function of external salt concentration. From the experimental data, and comparison with scaling and mean-field theories, we conclude that grafted HA films are well-described as a polyelectrolyte brush. Addition of cartilage proteoglycan aggrecan induced a drastic increase in thickness and resistance to compression. The novel combined AFM/RICM setup can serve as a powerful tool to quantify the mechanical properties of soft hydrated biopolymer films with precise control of probe sample separation.

1011-Pos Board B797

Raman Spectroscopy and Imaging of Biomolecules using Targeted Nanoparticles

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Abstract:
Optical microscopy of biological systems is useful for detecting various structures with varying chemical or structural contrasts. In the past, fluorescent tags have been useful as imaging probes of biomolecules. An alternative is to use optical properties of nanoparticles for contrast and detection. The local electromagnetic fields gained from the excitation of conduction band electronics of metal nanostructures can be used to enhance Raman scattering from molecules in close proximity. This effect, the electromagnetic enhancement responsible for surface-enhanced Raman scattering (SERS), thus provides a sensitive probe of chemical environments. We have coupled tip-enhanced Raman scattering (TERS) with nanoparticle probes to obtain chemical, structural, and spatial information simultaneously. In protein-ligand interactions, our results show signal enhancements from both the ligand, bound to a nanoparticle probe, and the target protein, thus demonstrating this environmental sensitivity. We are exploring these effects to distinguish the differences between the wild type and mutant proteins, as well as investigating intact cell membranes.

1012-Pos Board B798

Fast and Accurate FRET Quantification through Computation of the Minimal Fraction of Donor (mf_D) from TCSPC Data

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The study of the spatial and temporal dynamics of molecular interactions using FRET-FLIM is often compromised by the large number of photons required to fit the multiple-lifetime decay of the donor population in each pixel of an image. Long acquisitions prevent interacting dynamics to be detected in an image, while the use of high excitation intensities causes bleaching or unexpected cell responses. The computation of the minimal fraction of donor molecules (mf_D) undergoing FRET is a non-fitting approach that allows quantification of molecular interactions where the complexity associated to fitting a fluorescent decay with fretting and non-fretting components hampers quantification.

We have established the experimental conditions on which quantitative FRET analysis based on mf_D computation is preferable to fitting the fluorescent decay to a model with a weighted non-linear least-squares algorithm. The accuracy of the quantifying parameters as a function of the fraction of donor molecules involved in the interaction and the number of photons was studied for both fitting and non-fitting strategies using simulated TCSPC data that had been validated against actual experiments with FRETting constructs of fluorescent proteins (mTFP1-YFP) expressed in living cells.

In summary, the validity of the non-fitting minimal fraction of donor computation (mf_D) strategy for quantification of TCSPC FRET experiments is demonstrated not only for cases when fitting strategies fail due to the complexity of the decay, but also for simpler models when the number of detected photons is small. The conditions on which quantitative mf_D analysis of FRET experiments allows faster acquisitions than fitting strategies have been established. This approach is well suited for imaging protein interactions in living cells as faster acquisitions result in better resolved spatio-temporal dynamics.